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STATE UNIV FORT COLLINS DEPT OF MICROBIOLOGY C D BLAIR
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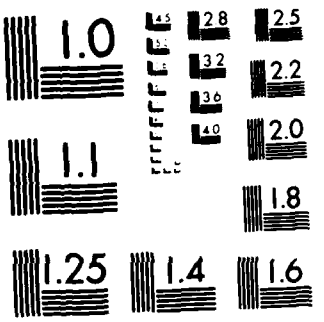

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Report Number 1

(1)

REPLICATION OF JAPANESE ENCEPHALITIS VIRUS

Biannual Summary Report

by
Carol D. Blair, Ph.D
Department of Microbiology

December 10, 1980

Supported by
U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-78-C-8047

Colorado State University
Fort Collins, Colorado 80523

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1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* Replication of Japanese Encephalitis Virus		5. TYPE OF REPORT & PERIOD COVERED Annual 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR*(s)* Carol D. Blair, Ph.D.		8. CONTRACT OR GRANT NUMBER*(s)* DAMD17-78-C-8047
9. PERFORMING ORGANIZATION NAME AND ADDRESS Colorado State University Fort Collins, Colorado 80523		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS10.AA.064
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE December 1980 13. NUMBER OF PAGES 26
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT *(of this Report)* Approved for public release; distribution unlimited		
17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*		
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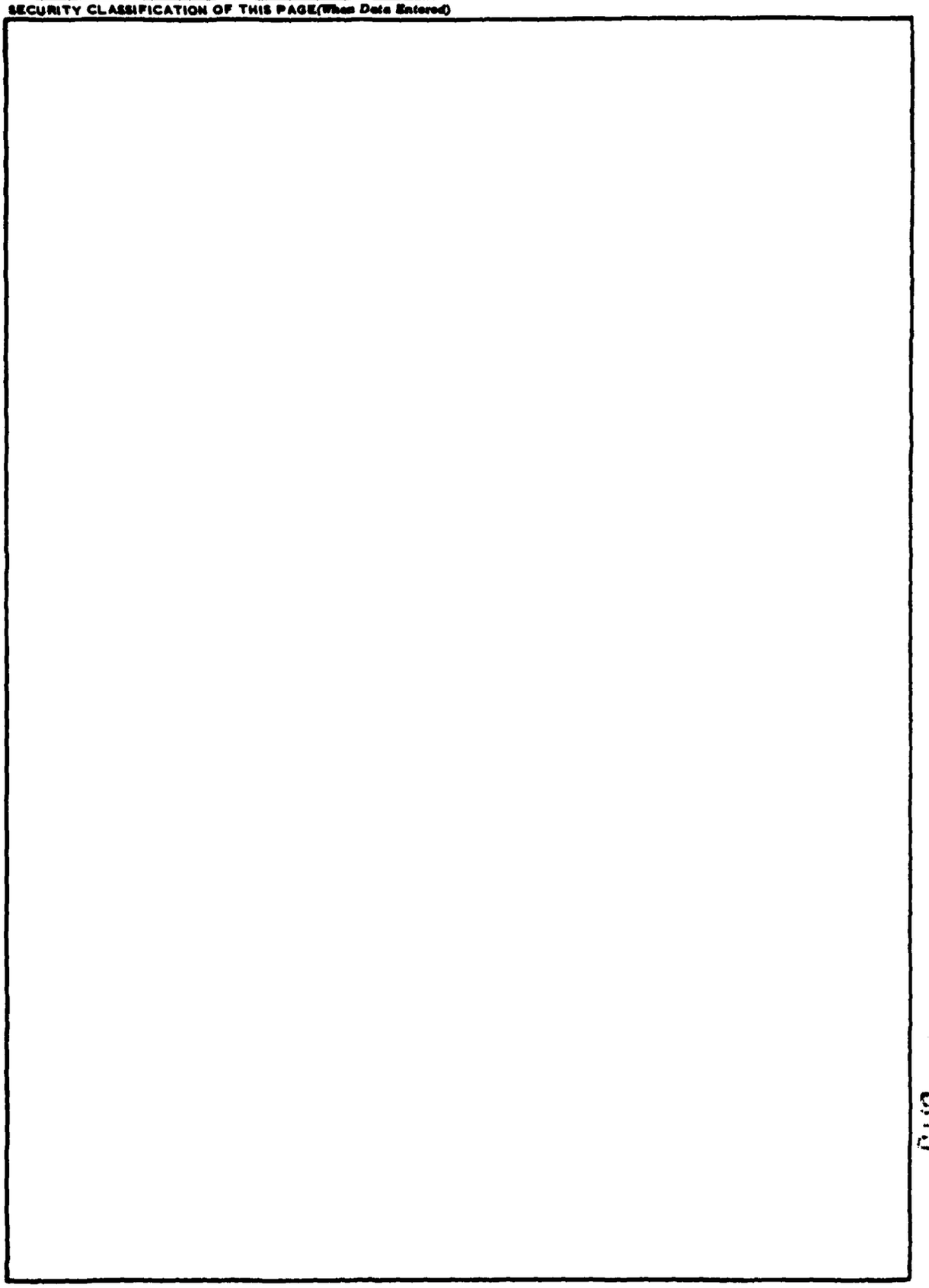

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ABSTRACT

The purpose of the project is to elucidate the replication of the flavivirus Japanese encephalitis virus (JEV) by exploring the following aspects: (1) RNA structure, sites and mechanisms of replication; (2) functions of virus-specific RNA's, strategy of protein synthesis; (3) induction, isolation, and characterization of temperature sensitive mutants; (4) studies of virus-cell interactions in persistent infections.

Virion RNA was determined to be single-stranded, with positive polarity after experiments which determined its base composition and infectivity. Its sedimentation coefficient was designated 40S since electrophoretic mobility was slightly greater than that of the alphavirus Venezuelan equine encephalitis virus. Preliminary chromatographic investigations utilizing ^{32}P-labeled virion RNA suggest that it possesses a 5'-cap with the structure m^7GpppG. Virion RNA in particles released from persistently infected cultures was indistinguishable from that of acute infections.

Virus-specific intracellular RNA was examined by sucrose gradient sedimentation and agarose gel electrophoresis. Both methods showed four size classes: 40S, 30S, 22S, 15S. Electrophoresis after glyoxal denaturation showed that 30S was a conformational variant of 40S (virion) RNA. The 22S component was apparently a partially double-stranded replicative intermediate. The 15S RNA was electrophoretically separable into two components designated 15S and 12S. Pulse labeling of RNA during the eclipse phase (early) and the exponential phase (late) of replication showed that synthesis of the 12-15S species occurred early, whereas synthesis of 40S RNA was overwhelmingly predominant during exponential growth. Isolation of polysomes by Mg^{+2} precipitation demonstrated that 12-15S RNA was polysome-associated early and 40S was in the polysomal fraction late in infection. Chromatography on oligo(dT) cellulose showed that virion RNA lacked polyadenylic acid, but all intracellular species were partially polyadenylated. These data all suggest that the 12-15S RNA species as well as the 40S (virion) RNA serve as virus-specific messengers.

Infected cells were labeled with ^3H-uridine, homogenized, and their membrane fractions separated by sedimentation on a discontinuous sucrose gradient. Virus-specific RNA synthesis was localized on smooth intracytoplasmic membranes.

The possibility that virus replication was localized in or dependent on the cell nucleus was eliminated by the following experiments. There was no electron microscopic evidence of nuclear involvement. Inhibitors of DNA synthesis (mitomycin C) and cellular RNA synthesis (actinomycin D) did not significantly diminish virus yield. No virus-specific RNA synthesis was detected in the nucleus.

Electron microscopic examination of thin sections of infected cells suggested that viral envelopes were not acquired by budding, but that nucleocapsids and surrounding envelopes may be formed by condensation of virus-specific smooth membrane spheres.

Five stable temperature-sensitive (ts) mutants were isolated after mutagenesis during virus growth with 5-fluoruracil or 5-azacytidine.

Two different mammalian cell lines which were persistently infected with JEV were studied. Over 200 cells were cloned from these cultures and all but four were nonproducers of infectious virus and viral antigen. However, nonproducer cells synthesized small amounts of virus-specific RNA. Superinfection of nonproducer cells with a mutant of JEV resulted in replication of the superinfecting virus only. No successful method for induction of the persistent genome was found. Although indirect evidence was obtained for release of interfering particles by persistently infected cultures and clones, no new size classes of virus RNA could be demonstrated.

TABLE OF CONTENTS

1. Background

Flaviviruses comprise a large genus of the family Togaviridae with considerable serological relatedness and biological homogeneity. They have worldwide distribution and are responsible for frequent outbreaks or epidemics of serious disease syndromes including hemorrhagic fever and encephalitis. Flaviviruses are taxonomically grouped with alphaviruses because of similarities in ecology (most members of both genera are arthropod-borne) as well as molecular biology (similar virion morphology and genome size). Understanding of the structure and replication mechanisms of alphaviruses has advanced rapidly due to extensive studies on the non-pathogenic prototypes Sindbis virus and Semliki Forest virus. Comparatively little is established of the molecular biology of flaviviruses for several reasons: 1) All flaviviruses appear to be human pathogens and thus require special laboratory handling. 2) Most flaviviruses grow more slowly and give lower yields in cell culture than alphaviruses. 3) In further contrast to alphaviruses, flaviviruses do not shut off host macromolecular synthesis [although they normally produce cytopathic effects (CPE) within 2-3 days]; thus virus-specific components are difficult to distinguish from those of the cell. It is not surprising, therefore, that alphaviruses have come to be used as models for the entire Togavirus family (Pfefferkorn and Shapiro, 1974). However, it is becoming obvious that flaviviruses differ in several details of their structure and replication from alphaviruses, and our research to date has been directed at elucidating these details.

The relatively few data from flavivirus studies are difficult to reconcile into a clear picture of structure and replication. The virion RNA has molecular weight of Ca. 4×10^6, is single-stranded and infectious, and therefore of positive polarity (Blair, unpublished observations). It codes for 3 virion structural proteins and 4-6 non-structural proteins whose precise identities and functions are not clear. Replication of RNA is presumed to be through a double-stranded replicative intermediate which sediments at about 20S (analogous to alphaviruses). There are various contradictory reports about the number and sizes of other intracellular RNAs. Earliest reports described 40S, 22S, and possible 26S molecules like those found in alphavirus-infected cells (Stollar et al., 1967; Trent et al., 1969). Later, several other species around 30S and 10S were described (Zebovitz et al., 1972; Takeda et al., 1977). The most recent studies on flavivirus-specific RNAs examined synthesis during the late exponential period of the virus growth cycle (generally 18-24 hr PI), and concluded that the genome 40S, and its 22S replicative intermediate in minor amounts, were the only intracellular viral RNA molecules present (Boulton and Westaway, 1977; Wengler et al, 1978). We had noted earlier that cells either acutely or persistently infected with Japanese encephalitis virus (JEV) contained virus-specific RNAs sedimenting at about 40S, 30S, 22S, and 15S, although the 40S was most prominent during the period of maximum RNA synthesis and virus release 18-24 hr PI (C. Blair and C. Schmaljohn, unpublished observations). In our efforts to determine whether or not each of these was a unique

molecular species and if so, what was its probable role in replication, we recently decided to examine which of these molecular species were synthesized (and presumably functioned) during the eclipse phase of infection. We found that virus-specific RNA could be detected in infected cells by pulses of ^{3}H-uridine as early as 3-5 hr PI. All 4 size classes were present during the early eclipse phase, but subgenomic species were more prominent and relative proportions were quite different from late exponential phase (detailed in progress report). The same RNAs were also present in persistently infected cells in proportions which varied according to the extent of virus production (Blair and Schmaljohn, 1980). Interestingly, Andzhaparidze et al., (1979) reported similar patterns of RNA synthesis in cells persistently infected with or during the eclipse phase of acute infection with tick-borne encephalities virus, although the probable significance was overlooked.

Concentrating on the 40S molecule as the only viral mRNA, Westaway (1977) presented evidence for multiple internal translation initiation sites on the flavivirus genome--a unique replication strategy which would require no subgenomic mRNAs. Additional evidence for (Svitkin et al., 1978) and against (Wengler et al., 1979) this hypothesis has been presented and the question of number and sizes of flavivirus mRNAs remains open.

The site of viral RNA synthesis and a possible nuclear requirement for viral replication are also longstanding questions. A number of studies involving cell fractionation have suggested somewhat unconvincingly that viral RNA was synthesized in the nucleus (Takeda et al., 1977) or on the nuclear envelope (Zebovitz et al., 1974). More credible evidence for nuclear dependence was presented in the enucleation experiments of Kos et al., (1975). Our studies have sought to lay to rest the question of nuclear involvement by a combination of methods including fractionation, nuclear inhibitors, and, autoradiography (Leary and Blair, 1980b).

There are few reports of isolation of induced or spontaneous temperature-sensitive mutants of flaviviruses. This is due not only to the previously mentioned characteristics which render flavivirus research difficult, but also to further problems such as high reversion rates, high ratios of plating efficiency at nonpermissive relative to permissive temperature, and complex assay procedures (Tarr and Lubiniecki, 1976a, b). Nevertheless, the potential usefulness of stable temperature sensitive mutants in studying the molecular biology of flavivirus replication and in developing preventive therapy for flavivirus disease (Eckels et al., 1976) suggest that this is an area of research worth pursuing.

It has been demonstrated that flaviviruses can persist in humans following vaccination (Monath, 1971) or natural clinical infection (Ishii et al., 1968). In view of the growing realization of the role of persistent viruses in some chronic human disease (Nathanson, 1977), it seems important to study mechanisms of viral persistence and long-term effects on host cells. We have established persistent infections of JEV in mammalian cell cultures which are normally subject to virus-mediated cytopathogenicity (Schmaljohn and Blair, 1977; 1979). Examination of these cultures has revealed a great deal about the varied mechanisms of viral persistence and cell sparing, as well as providing valuable comparative tools for the study of acute, lytic infections.

The combination of projects which have been supported under our current contract have significantly advanced our understanding of JEV replication. Progess in our perception of patterns of RNA synthesis has been good: we have a reasonable understanding of the number and size of virus-specific RNA classes and their probable functions. Work on cellular sites and kinetics of virion synthesis and assembly is essentially complete. Isolation of temperature-sensitive mutants has progressed more slowly that hoped, partly because of the intractability of the virus and partly because of a change in the graduate research assistant assigned to the project. The persistently infected cultures have provided a wealth of unanticipated information which has bolstered all aspects of research on replication of Japanese encephalitis virus.

Following is a detailed account of our progress between June 1978 and February 1980. The report is arranged and titled in parallel with the original request in order to facilitate comparison.

2. Characterization of structure and functions of JEV virion RNA.

Virion RNA has been designated 40S by comparison of its sedimentation rate in sucrose gradients to 28S ribosomal RNA and because of its electrophoretic mobility is slightly greater than that of the alphavirus Venezuelan equine encephalitis virus (VEEV), which is designated 42S (Blair and Schmaljohn, 1980). Much of our work on "virion" RNA has utilized 40S RNA extracted from infected cells for several reasons: 1) Study of interrelationships between the entire intracellular RNA population has given a more accurate and complete picture of replication. 2) Due to low yields of released virus in infected vertebrate cell cultures, intracellular 40S RNA is more abundant than virion RNA. Evidence that intracellular 40S RNA is identical to virion RNA comes from their identical sedimentation rates in sucrose gradients and the fact that both are infectious (C. Blair, unpublished results). However, some points of divergence between intracellular and extracellular RNAs have emerged: 1) There is a slight difference in electrophoretic mobility in agarose gels. 2) The question of whether or not some intracellular 40S RNA is polyadenylated is not resolved (see later), and leaves open the possibility that an intracellular subpopulation(s) exists which differs from virion RNA. Future work will directly examine the relationship of these two RNA populations.

a. Methylation. Determination of presence of 5'-methylated caps on virus-specific RNAs should identify mRNAs. Internal methylation patterns will aid in localization of sites of synthesis (see 1978 proposal). In order to determine if virus-specific RNAs have methylated 5'-caps, JEV-infected cells were labeled with ^{32}P-orthophosphate in the presence of 5 μg/ml actinomycin D (act D) from 12-24 hr post infection (PI). RNA was extracted, separated on a sucrose gradient, and each size class was hydrolyzed to completion with RNase T2. The entire hydrolysate was fractionated by paper electrophoresis (Pettersson et al., 1977), thus minimizing losses of material due to multistep chromatographic procedures. Putative caps were located near the origin by autoradiography, eluted, and treated with alkaline phosphatase and P1 nuclease. Products were identified by two-dimensional thin-layer chromatography. Preliminary results indicate that both 40S and 15S RNAs have

caps with the probable structure m^7GpppG. These results will be re-examined, since both dengue (Cleaves and Dubin, 1979) and West Nile virus (Wengler, Wengler, and Gross, 1978) intracellular 40S RNAs are reported to have the type I cap structure $m^7GpppAm$.

Labeling with ^3H-methyl-methionine has not yet been attempted. The results of Cleaves and Dubin (1979) with this procedure are of interest, since they found that dengue virus 40S RNA has little or no internal methylation, leading to the inference of a cytoplasmic site of synthesis (see 1978 proposal).

Our preliminary results point to a messenger role for both 40S and 15S RNAs and, considering the infectivity of virion RNA, suggest that 15S may be a subgenomic set.

b. Comparison of virion RNA from acute and persistent infections. Biological and indirect biochemical evidence indicates that virus released from persistently infected cultures is defective and interferes with the replication of standard virus (Schmaljohn and Blair, 1977; Schmaljohn, Happ, and Blair, 1980). Experiments comparing virions and virus-specific RNA from persistent infections to those from acute virus infections were designed to determine if defectiveness of the former is due to a deletion or some other size alteration in virion RNA. However, no size differences or new RNA size classes could be detected in virion RNA or intracellular virus-specific RNA from persistent infections by sedimentation and electrophoretic analyses under denaturing or non-denaturing conditions. Both virus producer and non-producer cell clones were examined, and whether superinfected or not, they failed to reveal other than the four RNA classes described below, although the distribution of label in RNA was reflective of the state of virus production (see below). These experiments suggest that RNA alterations may be in the form of point mutations, very small deletions, or if large deletions, are accompanied by insertion of cellular or redundant viral information. Two dimensional fingerprints of RNase T1-generated oligonucleotides are now being prepared from RNA species from persistently infected and acutely infected cultures to help resolve this question.

3. Synthesis and function of virus-specific intracellular RNA.

Recent experiments have clarified our understanding of the number and interrelationships of intracellular virus-specific RNAs, and will be described in a manuscript now in preparation (Blair and Schmaljohn, 1980). Cultured cells lytically infected with JEV and labeled with ^3H-uridine or ^{32}P-orthophosphate in the presence of act D during the period of maximum virus-specific RNA synthesis (18-30 hr PI) reproducibly yield four sedimentation size classes of RNA: 38-40S (40S), 30-35S (30S), 20-26S (22S), and 12-15S (15S). Up to 50% of the material in the 22S region is RNase-resistant. If extraction procedures minimize degradation of single-stranded RNA (dissolution in 1% SDS with or without proteinase K, extraction with cold phenol or phenol:chloroform), the vast preponderance of virus-specific RNA observed during this late exponential period sediments at 40S, making other classes appear insignificant (Boulton and Westaway, 1977; Wengler et al., 1978). If each sedimentation class is separately analyzed by electrophoresis in one

lane of a 1% agarose slab gel, it is seen that the electrophoretic mobilities of the 40S and 30S classes are identical, suggesting that they are conformational variants of the same molecule. In agreement with its partial RNase resistance, the 22S RNA remains near the origin, indicating that it has extensive secondary structure typical of a replicative intermediate, which sterically hinders its entry into the gel. The 15S RNA yields two distinct bands (designated 12S and 15S) with mobilities slightly greater than cellular 18S rRNA (Fig. 1).

Fig. 1. Agarose gel electrophoresis of JEV specific RNA

Irreversible denaturation of RNA by reaction with glyoxal in the presence of dimethyl sulfoxide (DMSO) destroys secondary structure so that electrophoretic mobility is determined solely by molecular size (McMaster and Carmichael, 1977). The 40S/30S, 15S, and 12S species when so denatured have lower electrophoretic mobilities, but retain their relative positions in the gel, confirming the presence of three distinct single-stranded virus-specific RNAs. If the 22S class is a replicative intermediate, it would be expected to yield 40S upon denaturation, yet even after reaction with glyoxal, most of the 22S remains at the top of the gel and a small proportion migrates to a position between cellular 28S and 18S. Todd and Martin (1979) reported that picornavirus replicative intermediates reannealed very quickly after DMSO denaturation, and such may be the case here. Further experiments will be conducted to resolve the questions of the nature of the 22S RNA and its metabolic role in replication.

During the late exponential period 18-24 hr PI, virus production has reached a maximum and 40S RNA synthesis would be expected to predominate. To determine if the smaller single-stranded RNAs were synthesized more abundantly during the eclipse period, and thus aid in defining their role in replication, 2-hr pulses of ^{3}H-uridine were given to infected cells, beginning 3 hr PI. In contrast to the pattern at 18-24 hr PI, in which 75% of label is incorporated into 30-40S RNA (Fig. 2C), during a pulse of ^{3}H-uridine given from 3-5 hr PI, 42% of label appears in 15S and only 8% in 40S and 24% in 30S (Fig. 2A). A pulse of uridine label given 12-14 hr PI produces an intermediate pattern, with approximately equal amounts of label entering 40S and 15S RNAs (Fig. 2B).

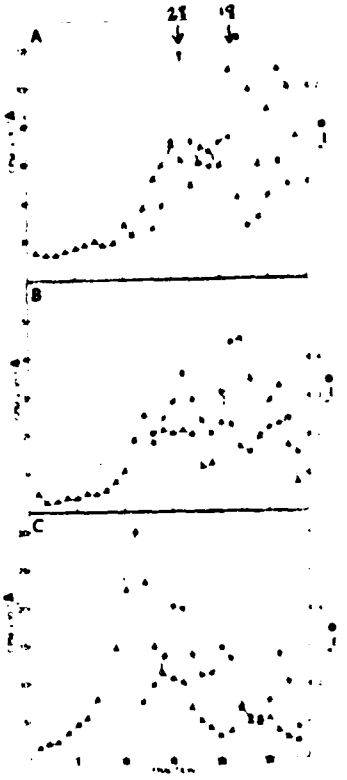

Fig. 2. Sucrose density gradient sedimentation of JEV-specific RNAs

These kinetics were observed when cells were infected at a multiplicity of infection (MOI) of 1-10. Lower MOIs resulted in delayed kinetics, but the order of emergence of virus-specific RNAs was the same. The

pattern was also unchanged in cells of several vertebrate species and with two different virus strains (Nakayama and M-1/311).

Most eukaryotic mRNAs are characterized by a polyadenylic acid [poly(A)] segment at their 3'-terminus. To examine JEV-specific RNAs for this property, and thus further define their role in replication, their ability to bind to an oligo(dT)-cellulose column was determined. This method was chosen after previous experiments with poly(U)-Sepharose columns gave false high estimates of poly(A) content apparently due to non-specific binding and inability to effect quantitative elution of sample from this medium.

Virion RNAs of JEV, VEEV, and vesicular stomatitis virus (VSV) (positive and negative controls, respectively) were labeled with ^{3}H-adenosine. Affinity chromatography of the purified 40-45S RNAs from each virus gave the following results:

Table 1. Oligo(dT) chromatography of virion RNAs

Virus	% RNA Bound
JEV	10.6
VEEV	77.1
VSV	8.0

The conclusion was that JEV-RNA, like VSV-RNA, has no significant polyadenylation.

Intracellular JEV-specific RNAs were similarly labeled, separated by sedimentation, and their binding to oligo(dT) compared to the 42S and 26S intracellular mRNAs of VEEV with the following results:

Table 2. Oligo(dT) chromatography of intracellular RNAs

RNA	% Bound
VEEV 26S	58.3
VEEV 42S	68.1
JEV 15S	29.8
JEV 22S	28.2
JEV 30S	26.7
JEV 40S	33.6

These results are somewhat ambiguous and reminiscent of findings for putative mRNAs of caliciviruses (Black et al., 1978) and coronaviruses (Lai and Stohlman, 1978). Similar levels of binding for flavivirus-

specific RNAs were found by Andzhaparidze et al., (1979) and Takegami and Yasui (1979). However, unlike Andzhaparidze et al., we did not observe higher levels of oligo(dT)-binding RNA in persistently infected cells. Failure of flavivirus mRNAs to bind to the same extent as alphavirus mRNAs could be due to one of the following reasons: 1) Presence of variable subpopulations within each size class; 2) presence of short (<50A) poly(A) segments on most flavivirus mRNAs; or 3) degradation of flavivirus RNAs before or during chromatography. Experiments to deal with the first and second possibilities are in progress. To determine if failure of most JEV-specific RNA to bind was due to degradation, both bound and unbound eluates were resedimented in sucrose gradients. Sedimentation rates had not changed for any fraction. Therefore, low levels of binding could not be attributed to RNA breakdown, and further studies will be required to answer the question of extent of flavivirus mRNA polyadenylation.

Our data on kinetics of synthesis and physical and chemical properties of virus-specific RNAs suggest that the two 15S moieties are subgenomic mRNAs which code for proteins required early in replication. The 30S form may also be an early messenger. Synthesis of the 40S mRNA/genome is greatly amplified at the time of maximum virus production. The 22S RNA appears to be a replicative intermediate, possibly for the synthesis of all single-stranded species, as in alphavirus-infected cells (Simmons and Strauss, 1972).

b. Cell fractionation. Cellular location of virus-specific RNAs is also an indication of their function. To determine whether the inferred mRNAs were polysome-associated, the magnesium precipitation method of Palmiter (1974) was used to isolate polyribosomes from infected cells at various stages of the replication cycle. This method avoids the problem of nucleocapsid contamination of polysomes cited by previous investigators (Naeve and Trent, 1978; Cleaves and Schlesinger, 1977). Pulse-labeled virus-specific RNA extracted from polysomes at 5 hr PI was predominantly (60%) 12-15S whereas at 24 hr PI, the vast majority of polysomal RNA was 40S. These findings reinforce the view that these are the predominant early and late JEV-specific RNAs, respectively.

Sites of RNA synthesis were determined by subjecting infected cells which had been pulse-labeled with ^{3}H-uridine to homogenization and sedimentation in a discontinuous sucrose gradient (Caliguiri and Tamm, 1970). Nuclei and six post-nuclear membrane-containing fractions were obtained and identified by electron microscopic examination and assay for the enzyme 5'-nucleotidase, which is confined to the plasma membrane.

Following is a tabulation of results from the cytoplasmic fractions labeled at 24 hr PI (for nucleus, see 4 below):

Table 3. Characteristics of cytoplasmic membrane fractions

Band No.	EM Appearance	5'-Nucleotidase Act. (U/mg protein)	Acid-Insol. CPM (infected)	Acid-Insol. CPM (control)
1	Smooth membrane myelin figures	319	3,612	840
2	Smooth membrane sheets	665	9,010	10,785
3	Smooth membrane vesicles, some virions	92	28,356	8,562
4	Smooth membrane spheres (SMS), virions, some RER	89	87,682	9,305
5	Virions, most RER convoluted masses of membranes	250	3,457	71,647
6	Mixture of above	443	1,078	2,450
Pellet	Ribosomes, few membranes	215	1,014	10,130

Conclusions are that plasma membrane comprises the bulk of bands 1 and 2, whereas bands 3 and 4, where late virus-specific RNA synthesis takes place, are derived from smooth intracytoplasmic membranes. The SMS, which are concentrated in fraction 4, are not seen in uninfected cells and are postulated to serve as intermediates in virion assembly (Leary and Blair, 1980a; see below).

c. Translation. Several preliminary attempts were made to translate virus-specific RNAs in a rabbit reticulocyte system supplied by Dr. R. S. Ranu. Incorporation of ^{35}S-methionine into products which separated into a number of discrete bands on PAGE was observed when 40S RNA was used as messenger. The 15S species also stimulated incorporation into acid-insoluble material. The products were not identified. When these experiments are resumed, the following problems will need to be addressed:

1) Production of sufficient virion RNA so that its products can be compared with those of intracellular 40S RNA.

2) Determination of optimal K^+ and Mg^{+2} concentrations to produce complete authentic products and eliminate premature termination.

3) Purification of intracellular virus-specific mRNAs free of cellular RNAs with similar sizes and properties.

4) Identification of products as virus-specific proteins.

Extensions of this important aspect of the project and proposed solutions to the recognized problems are under way.

4. Cellular site of virus replication, possible nuclear dependence.

The enduring question of whether flavivirus replication takes place in the nucleus or requires some nuclear function has been approached in several ways. Convincing evidence that any nuclear dependence was limited to early steps in replication was presented in the enucleation studies of Kos et al., (1975). Therefore, we emphasized early events in these studies.

Sequential occurrences in viral morphogenesis were examined by infecting Vero cells at MOI = 30-100, fixing and staining of the monolayer in situ at 3 hr intervals from 3 hr to 30 hr PI, and examining thin sections by transmission electron microscopy. No morphological changes in the nucleus or its envelope were observed at any time. Earliest visible cytoplasmic changes were seen at 15 hr PI when smooth membrane spheres (SMS) 70-140 nm in diameter began to accumulate within the cisternae of the rough endoplasmic reticulum (RER). Material enclosed within the spheres appeared as dots, rings, or fibrils. Mature virions could also be seen enclosed by ER or the membranes of the Golgi apparatus. No precursors identifiable as naked nucleocapsids were seen within cells. As time progressed, the number of ER-enclosed SMS increased and frequently were seen on the periphery of convoluted masses of membranes. Mature virions enclosed in vesicles or in channels of the ER appeared to leave the cell by exocytosis. No evidence of budding through intracytoplasmic or plasma membranes was seen.

We have put forward a model for virus morphogenesis (Leary and Blair, 1980a) in which the SMS, which appear to form by pinching or budding from ER, are precursors to the virus envelope. The model proposes that during their formation, the spheres incorporate envelope proteins synthesized on RER and enclose genome RNA (synthesized on SER) and capsid protein (synthesized in the cytosol). Through a condensation and maturation process which takes place within the cisternae of the ER and Golgi, virions are formed and remain membrane-enclosed until they are expelled from the cell. The convoluted masses of membranes accumulate as by-products of the condensation.

In an attempt to chemically determine the cellular origin of the viral envelope, phospholipid composition of purified virus was compared to that of the cellular membrane fractions separated on a discontinuous sucrose gradient as described in 2b above. Lipids were uniformly labeled by addition of ^{32}P-orthophosphate to culture medium from 1-48 hr PI. Comparison of the composition of membrane fractions from infected and uninfected cells showed that JEV infection did not alter lipid synthesis or metabolism. The following table shows percent of ^{32}P counts incorporated into each phospholipid class in each membrane fraction and in purified virus. Virus composition was not identical to any fraction, but most closely resembled fractions 3 and 4, which were composed largely of smooth and some rough intracytoplasmic membranes.

Table 4. Phospholipid distribution in celular membranes and virions

Band	Sphingo-myelin	Phosphatidyl-choline	Phosphatidyl-inositol	Phosphatidyl-ethanolamine	Cardio-lipin
1	--	73.3	--	6.7	30.0
2	10.5	64.1	11.1	7.2	7.2
3	6.1	54.6	12.7	17.3	4.3
4	3.6	60.2	11.5	18.0	5.7
5	11.7	67.0	9.8	4.5	7.0
6	10.3	65.8	11.9	6.6	5.5
JEV	0.1	49.0	12.9	28.3	4.4

Early biochemical events in virus replication were not visible in the EM and were probed with other methods. Cells were treated with inhibitors of various nuclear functions for periods before and afrer infection and their effect on virus yield at 24 hr PI was determined. Application of mitomycin C (at a concentration which inhibited cellular DNA synthesis by 95% and RNA synthesis by 30%) did not diminish virus yield. Four-hour pulses of act D (5 μg/ml, which inhibited cellular RNA synthesis by 97%) were applied from 4 hr before infection to 12 hr PI, then thoroughly washed from the cells. None caused more than a 2-fold variation in virus yield. These results suggested that neither nuclear DNA synthesis nor RNA transcription was required early in the JEV growth cycle. We believed that the apparent inhibition of flavivirus replication when act D was present in the medium throughout the replication cycle, or when nuclei were removed early, was probably an indirect effect due to loss of cell vitality and capability to support virus replication. We therefore designed an experiment to test this hypothesis. VEEV, an alphavirus with a one step growth cycle 12 hr in length (compared to 24 hr for JEV), was used as a control. Act D at 5 μg/ml, was added to culture medium at the times indicated below and remained until virus was harvested for assay, 12 hr PI for VEEV and 24 hr PI for JEV.

Table 5. Effect of actinomycin D on virus yield

Virus	Time of Act D Addition (hr PI)	Total Time of Treatment (hr)	Virus Yield (PFU/ml)
VEEV	--	0	1.5×10^7
VEEV	0	12	1.5×10^8
VEEV	-12	24	5.0×10^5
JEV	--	0	6.0×10^5
JEV	12	12	3.0×10^5
JEV	0	24	5.0×10^3

Our interpretation of these results is that VEEV replication is not inhibited and may even be enhanced in the presence of act D. However, VEEV yield is diminished when the virus is harvested from cells which have been treated for a total of 24 hr with act D, probably due to loss of cell viability. Therefore, the apparent act D inhibition of JEV replication is likely to be related to the long replication cycle and may be attributed to cell damage and not to a direct effect on virus replication. These experiments are detailed in Leary and Blair (1980b).

The possible nuclear role in virus replication was also examined by autoradiography of fixed, photographic emulsion-covered cells after very short pulses of ^{3}H-uridine in the presence of act D. Grains were counted over nucleus and cytoplasm of infected and similarly treated uninfected cells. Statistical analysis of results revealed that up to 9 hr PI, there was not a significant difference between JEV-infected and uninfected cells, probably due to the low level of early virus-specific synthesis. Post-eclipse period counts, however, revealed a cytoplasm/nucleus ratio of 2.6 for infected cells as compared to 1.1 for controls. Absolute counts demonstrated that all grains attributable to virus-specific synthesis were in the cytoplasm.

Similarly pulse-labeled cells were homogenized at 3 hr PI and their nuclei were separated from cytoplasm by centrifugation and thorough washing with NP-40. RNA was extracted from nuclear and cytoplasmic fractions and sedimented in a sucrose gradient. Total counts in cytoplasm were 100-fold more than those in nuclei. Cytoplasmic RNA sedimented in a typical "early" pattern with the predominant peak at 15S. Sedimentation of nuclear RNA displayed no distinct peaks.

Our conclusions based on these results are 1) that flavivirus replication is independent of any direct requirement for the cell nucleus, 2) that virus-specific RNA synthesis takes place in association with smooth intracytoplasmic membranes, 3) that membranes of the ER contribute to the formation of spheres (SMS) which are precursors of the viral envelope. Nucleocapsids are not formed as precursors to virions, but may come into existence by a condensation process inside SMS. The virus does not mature by budding.

5. Isolation and characterization of temperature-sensitive (ts) mutants of JEV

Our strategy has been to attempt to isolate a large number of (20-50) of stable mutants, induced by several different mutagens, before we began biochemical and genetic characterizations. To date we have 5 stable mutants, all induced with 5-fluoruracil (FU) or 5-azacytidine (AC). Each of these has been recloned from a single plaque at least 3 times. In acquiring these mutants, we picked and tested over 1000 plaques. Fifty or sixty of these appeared to be potential mutants at first assay, but almost all reverted to wild type (WT) efficiency of plating after 1-2 passages. Characteristics of the stable mutants are as follows:

Table 6. Characteristics of ts mutants of JEV

Designation	Titer at 33 C (PFU/ml)	Titer at 40C (PFU/ml)	Mutagen (conc)
ts-1	6×10^5	$<10^2$	FU (50 µg//ml)
ts-2	1.7×10^6	$<10^2$	AC (25 µg/ml)
ts-3	8×10^5	$<10^2$	AC (25 µg/ml)
ts-4	6×10^5	$<10^2$	AC (25 µg/ml)
ts-5	9×10^5	$<10^2$	FU (100 µg/ml
WT	1.5×10^6	9×10^5	----

Other mutagens have been employed, but an array of problems has hindered their usefulness. Treatment with N-methyl-N'-nitrosoguanidine (100 µg/ml, 15 min) did not cause a reduction in virus titer and none of the mutagenized virus was temperature-sensitive. Hydroxylamine (0.05 M, 20 min), even after dilution to 10^3, killed cells on which virus was plaqued, so effect on virus could not be determined. Nitrous acid (2M, 15 min) and ethyl methane sulfonate (2%, 15 min) totally inactivated virus, probably due to acid pH. Mutagenesis with these compounds will be attempted again, with appropriate alterations in times, concentrations, or pH. At the same time, we will begin to ascertain the phenotypes of the 5 stable mutants. If there are indications that they have lesions in different genes, we will assume that FU and AC, the successful mutagens, did not selectively mutate certain genome "hot spots" and we will continue to mutagenize with them. Our experience of high rates of reversion with potential JEV mutants gives confidence that the concentrations and exposures to FU and AC which we have used induce single point mutations. We may have to raise the concentrations, at the risk of multiple mutations, to achieve stability, and we must certainly consider the possibility that the mutants we have carry multiple genetic lesions.

6. Persistent infections of vertebrate cells by JEV

Our persistently infected mammalian cell lines are finding more usefulness than we anticipated in studies of RNA replication, (Blair and Schmaljohn, 1980), mechanisms of CPE, and genetic interactions, as well as mechanisms of viral persistence. Progress in the latter area is summarized here and in three publications (Schmaljohn and Blair, 1977; Schmaljohn and Blair, 1979; Schmaljohn, Happ, and Blair, 1980).

Attempts to isolate and characterize defective virus or its genome from persistently infected cultures by means which have been successful in other systems (differences in sedimentation rate or buoyant density of virus, size differences in RNA due to deletions) have not revealed differences between virus produced in standard and persistent infections (detailed in part 2). Virus concentrated from medium of persistently infected cultures does interfere with replication of WT virus,

however (Schmaljohn and Blair, 1977), and yield of WT virus used to superinfect carrier cultures is depressed as compared to infection of normal cells. Therefore, we feel that defective virus or some other interfering factor is present in the medium of carrier cultures.

In an effort to examine cellular properties which influence viral replication and persistence, we isolated over 200 cell clones from various JEV-persistently infected rabbit kidney (MA-111) and monkey kidney (Vero) cell cultures. Each of these was grown into a monolayer and passed at least 5 times, with assay for infectious virus (by plaque assay) and viral antigen (by immunofluorescence) at each passage. Only 4 of the 200 clones were positive for virus and viral antigen and these had characteristics very similar to parental cultures. The only evidence of viral persistence in non-producer clones was virus-specific RNA, which could be labeled in the prescence of actinomycin D.

Ten of the nonproducer clones were randomly selected for further study. All of these could be superinfected with WT virus without production of CPE, and all commenced virus production after superinfection (Schmaljohn and Blair, 1979). To determine whether virus which replicated as a result of superinfection contained the superinfecting genome or represented induction of the persistent genome, ts-2 was employed to infect the nonproducer cultures. All infectious virus released at the permissive temperature was ts, and none was released at the non-permissive temperature, indicating that only the superinfecting virus had replicated and the resident genome had not been induced. These ts-superinfected cultures are being maintained at the permissive temperature and monitored at intervals to determine if genetic interactions between the two types of JEV genomes or their gene products can be detected.

We believe that the cloning process selected for the healthiest cells, and that the above results indicated that the most stable virus-cell equilibrium state, in terms of cell survival, is that in which synthesis of viral structural components is minimal, although superinfection leading to virus production does not usually cause cell death. Whether the level of synthesis is controlled by viral or cellular factors has not been determined. These nonproducer cultures will be exploited in determining factors which contribute to viral CPE.

Other methods were sought to shift the virus-cell equilibrium in favor of either curing or cell killing. Treatment of persistently or endogenously infected cells with inhibitors of macromolecular synthesis has been reported to induce virus replication in other systems. However, 5-bromodeoxyuridine, act D, and cycloheximide failed to induce JEV production. Superinfection of persistently infected cultures at high MOI (= 10) in some cases caused complete cell killing, whereas in most cases it had no harmful effect. Addition of antiviral antiserum for several passages to medium of virus-producing carrier cultures resulted in a lower virus yield and low proportion of cells producing viral antigen, but did not cure the culture of viral RNA. In summary, we have not yet found any external factor which readily alters the virus-cell balance.

Our original impression was that during establishment of viral persistence, virus expression drops to the minimal level required to assure passage of the viral genome to each daughter cell and stabilizes. Continued passage of the nonproducer clones has shown that this is not always the case. Between the 20th and 50th passages, some clones began spontaneous virus production. In some cases, 100% of the cells in a culture released virus, whereas in most, only a minority of the cells shed virus. A few cultures died after commencing virus release, but most did not, and no correlation was seen between proportion of cells shedding virus and culture death. Some eventually ceased virus production, while others have continued for up to 20 further passages. Nonproducer clones synthesized virus-specific RNA in an "early eclipse" pattern (mostly 15S RNA) whereas after spontaneous or superinfection-induced virus release began, the RNA pattern was that of "late eclipse" (equal amounts of 15S and 40S).

Examination of nonproducer carrier cultures by EM revealed few of the ultrastructural changes associated with virus infection (see section 3). Producer cultures contained a number of cells with proliferating intracytoplasmic membranes, SMS, and membrane-enclosed virions, and so were indistinguishable from low MOI acute infections. Superinfected carrier cultures contained bizarre cells with cytoplasms completed filled with SMS, yet relatively few virions. It is hard to imagine such cells functioning normally, yet culture growth was indistinguishable from uninfected.

In summary, these observations do not allow us to form a coherent or all-inclusive hypothesis for the mechanism of persistence of a normally cytolytic virus. Indeed, they suggest several mechanisms probably exist. We believe that formation of a stable virus-cell complex usually derives from minimal viral expression, possibly through repression of synthesis of the viral genome and/or structural proteins. This repression is probably due to both viral and cellular factors: virus-directed production of interfering particles or molecules and inhibition by the cell of certain aspects of virus-specific transcription and/or translation.

6. Conclusions

On the basis of the information we have accumulated, we can propose the following scenario for the replication of JEV. It diverges from previously proposed strategies for flavivirus in invoking the use of multiple subgenomic mRNAs.

JEV attaches to a host cell receptor and enters the cytoplasm where, after partial or complete uncoating, the genome RNA associates with membranes of the ER. Cellular ribosomes translate the entire genome, probably as a polyprotein. Posttranslational cleavage produces several polypeptide products. One or more of those coded at the 3'-end are viral RNA polymerases (cf. Wengler et al., 1979; evidence that structural proteins are coded at 5'-end). The polymerase(s), which may be cis-acting, associate with a binding site at the 3'-end of the genome to

synthesize the full-length negative strand which will be a template in the RI. The RI is then used predominantly for synthesis of 12S and 15S (and perhaps 30S) mRNAs and these are translated to give more polymerase. Additional RIs are formed, on which 40S RNA synthesis now begins. The 40S RNA is a template for structural protein synthesis. Capsid protein, synthesized in the cytosol, begins condensation around the genome. The 2 envelope proteins are synthesized on and remain associated with RER. The RER which has been modified by association of virion structural proteins, pinches off to form SMS, which enclose genome and capsid protein and eventually condense to mature virions. Final glycosylation takes place in the Golgi, and exit is through the channels of the RER.

Admittedly this is highly speculative, and gap-filling and modifications will be necessary as we conduct the experiments described in our new proposal.

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Degrees completed under contract support

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